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13. ABSTRACT ***(Maximum 200 Words)*** We investigated neurotransmitters which play a role in conveying light information to the circadian clock in the suprachiasmatic nucleus (SCN). We studies the effects on SCN cell responses to light of classic small-molecule transmitters, such as glutamate, serotonin and acetylcholine, as well as a number of peptides. We showed that glutamate can affect SCN cell firing rates through both ionotropic and metabotropic receptors. We found that the peptides GRP and VIP also affect SCN in vivo with a pattern of temporal sensitivity similar to that of light. We studied how serotonin and melatonin alter photic responses of SCN cells, and showed that serotonin acts via a receptor that resembles the 5-HT7 subtype. We recently demonstrated differences in the responses to light of SCN neurons in nocturnal rodents compared to those is degus, a diurnal/crepuscular rodent. In vivo and in vitro studies suggest the possibility that the inhibitory neurotransmitter GABA is found in the retinal entrainment route in both types of mammals, along with the excitatory transmitter glutamate. To facilitate our behavioural studies, we developed a data analysis system for dealing with activity rhythm data collected by computer.

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Title: Neurophysiological and behavioral analysis of circadian rhythm entrainment

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Principal Investigator: Benjamin Rusak

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ABSTRACT

We investigated neurotransmitters that play a role in conveying light information to the circadian clock in the suprachiasmatic nucleus (SCN). We studied the effects on SCN cell responses to light of classic small-molecule transmitters, such as glutamate, serotonin and acetylcholine, as well as a number of peptides. We showed that glutamate can affect SCN cell firing rates through both ionotropic and metabotropic receptors. We found that cholinergic agents act via postsynaptic receptors of the muscarinic subtype but that nictonic receptors in the SCN are largely presynaptic and their effects are mediated via modulation of glutamate release. We studied how serotonin alters photic responses of SCN cells, and showed that it acts via a receptor that resembles the 5-HT_7 subtype. We recently demonstrated differences in responses to light by SCN neurons in nocturnal rodents compared to those in degus, a diurnal/crepuscular rodent. In vivo and in vitro studies suggest the possibility that the inhibitory neurotransmitter GABA is found in the retinal entrainment route in both types of mammals, along with the excitatory transmitter glutamate. To facilitate our behavioral studies, we developed a data analysis system (Circadia) for dealing with activity rhythm data collected by computer.

A. Photic Responses and Firing Rates in a Diurnal Rodent

The characteristics of light-responsive SCN cells in cats and nocturnal rodents were documented in studies in the 1970s and 1980s. These cells showed sustained responses during lengthy retinal light exposures, had large receptive fields, high thresholds, and were more often light-activated than light-suppressed. From 65-85% of photically responsive cells were activated by retinal input across studies using light, optic nerve stimulation, different anesthetic conditions, and different methods of recording SCN cells (Nishino et al., 1976; Sawaki, 1979; Miller et al., 1987; Meijer and Rietveld, 1989; Meijer et al., 1996). In the one previous study of a diurnal rodent, the ground squirrel, *Spermophilus tridecimlineatus*, these characteristics differed in that thresholds were much higher than even those reported for rats and hamsters, and more cells were light-suppressed than light-activated (Meijer et al., 1989).

The interpretation of these observations was confounded by the fact that the ground squirrels were wild-caught rather than laboratory-bred and because they had to be anesthetized using a barbiturate anesthetic rather than urethane, which had been used in most previous *in vivo* studies in nocturnal rodents. Thus, threshold and response-type differences could be attributable to the anesthetic or to the previous history of bright, natural-light exposure in these squirrels. We recently re-examined this issue using degus, which are predominantly diurnal South American hystricomorph rodents. While degus show strong crepuscular modulation of their temperature and sleep rhythms, and modify their rhythms in response to some environmental conditions, in most previous studies of their rhythms, as well as in field reports, they are predominantly diurnal, with crepuscular modulation (Lee and Labyak, 1997; Kas and Edgar, 1998).

The degus used in our study (Jiao et al., 1998) were laboratory-bred and could be anesthetized with urethane, so we conducted studies in parallel on rats and degus using the same

recording situation and methods. We found that SCN cells in degus were as likely as those in rats to show sustained photic responses (~25% of cells). However, as in ground squirrels, more photically responsive cells were light-suppressed than light-activated in degus. In fact, among light-responsive SCN cells, 85% were *activated* by light in rats, while 73% were *suppressed* by light in degus. These results confirm the findings from ground squirrels recorded under different conditions, and suggest that predominant diurnality is associated with a different pattern of light-responsiveness among SCN cells than that observed in nocturnal species.

We incidentally observed other differences between rats and degus. In rats, baseline firing rates of photically responsive SCN neurons were similar during day and night phase recordings. In degus, these cells showed higher firing rates during the night, but the difference was not statistically significant. Among photically unresponsive cells (~75% of all cells recorded in both species), there was a significantly higher firing rate during the subjective day than during the subjective night in rats. This observation is consistent with a wealth of previous data showing that the activity of SCN cells in rats oscillates and reaches a peak during the subjective day (e.g., Schwartz et al., 1980; Prosser et al., 1993). However, in degus, photically unresponsive neurons did not show a similar daytime peak, and firing rates did not differ between day and night phases.

These results suggest two conclusions: 1) The major contributor to the circadian firing-rate rhythm of rat SCN cells is the large population of photically unresponsive cells, while photically responsive cells show no significant daily rhythm of baseline firing rate under these conditions. 2) In degus, neither population of cells shows a significant baseline firing-rate rhythm. In fact, the tendency among the relatively small population of photically responsive cells was to show higher rates during the night. A larger number of cells might reveal a statistically significant rhythm peaking in the night, but this hypothesis remains to be tested.

Before concluding that these differences are related to differences in activity type, it should be noted that one previous study of multiple-unit firing rates in a diurnal chipmunk reported a daytime peak (Sato and Kawamura, 1984). On the other hand, studies of 2-deoxyglucose (2-DG) uptake, as a marker of SCN metabolic activity, yielded conflicting results. While diurnal squirrel monkeys showed a daytime peak of 2-DG uptake (Schwartz et al., 1983), diurnal 13-lined ground squirrels did not show a rhythm of 2-DG uptake in a study that did observe daytime peaks in both rats and Syrian hamsters (Flood and Gibbs, 1982). Interpreting these results is complicated by our ignorance of how population-based rhythms of firing-rate and glucose-uptake are related to each other and to the activity of single SCN neurons. It is possible that different cells contribute to these rhythms, or, for example, that increased glucose uptake in some cells is associated with release of inhibitory transmitters and depressed activity in other cell populations. Further analysis is needed of the patterns of activity of individual photically responsive and unresponsive cells in both nocturnal and diurnal species.

Another possibility that we have explored is that some suppressions of SCN cell firing caused by retinal illumination reflect release of an inhibitory transmitter in the RHT. We tested this hypothesis using a brain slice preparation containing the SCN and the optic nerves. Brief stimulation of the optic nerves evoked both activations and suppressions of SCN cell firing rates in both rats and degus. Interestingly, the larger proportion of light-suppressed cells observed in vivo in the degu SCN was matched by a larger proportion of cells being suppressed by optic nerve stimulation in the slice preparation. Activations in rats were blocked by bathing the slice in NMDA antagonists, such as APV, while suppressions were blocked by application of the $GABA_A$ antagonist bicuculline.

The latter effect could have reflected activation via release of RHT glutamate of GABA-containing SCN neurons, resulting in the secondary release of GABA and thus inhibition of the target neuron being recorded. If this were the case, then suppressions of cell firing in response to optic nerve stimulation would be blocked either by application of the GABA antagonist bicuculline or by application of the glutamate antagonist APV. In preliminary studies, we have demonstrated that some suppressions evoked by optic nerve stimulation can be blocked by bicuculline but not by APV (Jiao and Rusak, 1999, unpublished observations). These results imply that there is no intervening release of glutamate, but that some RHT fibers release GABA directly. This novel hypothesis needs to be pursued with additional studies.

B. Glutamate and the RHT

Glutamate is the principal excitatory transmitter in many parts of the nervous system. Since glutamate is found in RHT terminals (Castel et al., 1993), and blockade of glutamatergic receptors can reduce light-evoked rhythm shifts and light effects on SCN cells (Abe et al., 1992; Colwell and Menaker, 1992), it, or a closely related amino acid, is a likely candidate for conveying photic information to SCN cells. It has been implicated as the principal neurotransmitter in the retinohypothalamic tract (RHT), mediating photic input from the retina to the SCN (Kim and Dudek, 1991), and probably to the intergeniculate leaflet (Stamp et al., 1997; Zhang & Rusak, unpublished). There are numerous glutamate receptor configurations made up of multiple subunits, but the principal types of receptors are those coupled to ion channels (ionotropic) and those coupled to G proteins (metabotropic). Ionotropic receptors can be divided into those preferentially binding N-methyl-D-aspartate (NMDA) and those preferentially binding kainate or quisqualate (non-NMDA receptors). Both NMDA and non-NMDA receptors have been reported to play a role in conveying photic information to the SCN (see below). While metabotropic receptors have been shown to mediate neurophysiological effects on SCN cells, their role in rhythm phase-shifting is less certain.

Application of glutamate or NMDA to SCN cells excites most cells tested in vivo, but a minority of SCN cells have been suppressed by glutamate (Nishino and Koizumi, 1977), and by optic nerve stimulation (Nishino et al., 1976). A mechanism for these effects is suggested by evidence that firing-rate suppressions in response to bath application of NMDA in a slice preparation may be mediated transsynaptically via glycinergic interneurons (Schmahl and Böhmer, 1997). While an early report indicated that glycine affected few SCN cells in a slice preparation (Shibata et al., 1983), more recent data indicate that glycine may be quite important. Levels of glycine release in the SCN are reported to be 10-fold those of aspartate and glutamate (Shinohara et al., 1998), and glycine affects chloride channels in dissociated SCN cells studied using whole-cell, voltage-clamp recordings (Ito et al., 1991). These results suggest a strychnine-sensitive glycine receptor plays an important role in regulating SCN cellular activity.

Short-latency responses to light input are mediated by rapidly acting, non-NMDA receptors (Kim and Dudek, 1991), but natural photic stimuli are slowly changing and sustained relative to the rapidity of neural transmission. Even nocturnal rodents (which generally avoid extended light exposure), when self-selecting their own photoperiods are exposed to light pulses of several minutes duration (DeCoursey, 1986). Under laboratory conditions, the hamster circadian system can also functionally integrate light input over several minutes (Takahashi et al., 1984). Electrophysiologically, photic activation of SCN cells via retinal input can be sustained for many minutes without decrement (Meijer and Rietveld, 1989). A receptor with the slow-acting, sustained character that fits these features is the NMDA receptor, which has therefore been proposed as a likely mechanism for conveying long-lasting photic input to SCN cells (Colwell et al., 1990).

We have studied several features of SCN cell physiology related to glutamatergic transmission and other aspects of the RHT. We performed a mapping study of glutamate receptor subunits in the hamster SCN and described the distribution of subunits contributing to both non-NMDA and NMDA receptors throughout the SCN and IGL (Stamp et al., 1997). It should be recognized that in addition to the RHT the SCN receive a number of afferents that appear to be glutamatergic (De Vries and Lakke, 1995; Moga and Moore, 1996). Thus, some of the receptor populations identified may not be targets of retinal afferents, and pharmacological manipulations that affect glutamatergic receptors in the SCN cannot be assumed to be affecting only RHT input.

A number of our studies of the RHT have revealed some unexpected complexity in its function and structure. Early studies of the role of glutamatergic transmission in mediating photic effects on immediate-early gene (IEG) expression in SCN cells reported that blockade of either NMDA or non-NMDA receptors could prevent light-evoked IEG expression in large portions of the SCN (Abe and Rusak, 1994; Abe et al., 1992), and reduce phase-shifting effects of light (Colwell and Menaker, 1992). These results are consistent with evidence from neurophysiological studies (Kim and Dudek, 1991; Aggelopoulos and Meissl, 1998) that both receptor classes contribute to RHT input to the SCN. However, we also found that these antagonists spared a region in the dorsolateral SCN that continued to express IEGs in response to nocturnal illumination (Abe et al., 1992).

In a subsequent study, we demonstrated that this same dorsolateral region showed IEG expression in response to electrical stimulation of the IGL region in the absence of light (Abe and Rusak, 1992). This effect of stimulation was abolished by optic nerve transection, implying that it was mediated by retrograde activation of retinal ganglion cells projecting to both the IGL and SCN (Treep et al., 1995). This finding, therefore, implied the existence of an RHT component, collateral to the projection to the IGL, localized to the dorsolateral SCN, and using a transmitter/receptor combination unaffected by the antagonists that block NMDA and non-NMDA ionotropic receptors.

We subsequently investigated the responsiveness of SCN cells in a hamster brain slice preparation to NMDA and to the metabotropic receptor agonist 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD). We found that cells in the ventral SCN were responsive to both NMDA and 1S,3R-ACPD. Responses to the metabotropic agonist were brisk and potent, with short latency and prolonged recovery to baseline. Cells in the dorsal SCN were also responsive to 1S,3R-ACPD, but in this case, latencies were long, thresholds high, and activation was rapidly reversed. The effects of 1S,3R-ACPD, but not NMDA, were antagonized by application of the phenylglycine derivative RS-α-MCPG, which acts as a metabotropic receptor antagonist (Scott and Rusak, 1996). We have subsequently studied the effects of metabotropic agonists and antagonists on circadian phase in hamsters. The most interesting finding was that pretreatment with either RS-α-MCPG or dizocilpine (MK-801, a non-competitive NMDA receptor antagonist) could each reduce the phase-shifting effect of a light pulse by about 50% (Scott et al., unpublished). These findings suggest that both ionotropic and metabotropic receptors mediate photic effects on SCN cells. 1S,3R-ACPD injected into the SCN did not cause phase shifts on its own (Scott et al., unpublished), while NMDA does so (Mintz and Albers, 1997); thus the role of metabotropic receptors may depend on simultaneous activation of other receptors. Reports on these studies are in preparation.

Another set of studies were aimed at the mechanisms by which histamine acts in the SCN to affect circadian rhythms (Cote and Harrington, 1993). We demonstrated that the weak histamine effects observed neurophysiologically on SCN cells in a hamster brain slice preparation were not mediated via classical histamine receptors. Histamine injections into the SCN in vivo also had very weak or no phase-shifting effects. However, we had evidence that histamine could alter SCN cell responses to NMDA, suggesting a possible modulatory role for histamine acting on an amine regulatory site in the NMDA receptor complex (Scott et al., 1995; 1998). Although few histamine-immunoreactive fibers were found in the hamster SCN, it may be that histamine acts as a neuromodulator of glutamatergic transmission via its effects on the NMDA receptor, an hypothesis supported by evidence from another laboratory (Eaton et al., 1996).

We do not know by what mechanism cells in the SCN are suppressed by retinal illumination. The evidence that glutamatergic neurotransmission mediates RHT effects on SCN cells does not provide an obvious mechanism, since glutamate is an excitatory transmitter. Activation of different classes of metabotropic receptors can modulate synaptic transmission and cellular physiology in diverse ways, including hyperpolarizing cells (Nusser et al., 1994; Colwell and Levine, 1994; Rainnie et al., 1994; Gereau and Conn, 1995). However, the one metabotropic agonist studied so far activated SCN cells (Scott and Rusak, 1996), and there is no evidence that glutamate can act via metabotropic receptors directly on SCN cells to suppress firing. One study has described cells in the rat SCN that were suppressed by bath application of NMDA (Schmahl and Böhmer, 1997). For most of these cells, pretreatment with strychnine, which blocks receptors for the inhibitory amino acid glycine, prevented the suppression by NMDA (Schmahl and Böhmer, 1997). This observation suggests that NMDA was activating a glycinergic interneuron, which in turn inhibited the cells being recorded. This finding suggests a mechanism by which light can suppress firing of SCN cells indirectly following the release of glutamate from RHT terminals.

C. Acetylcholine (ACh)

The role of ACh in the rodent circadian system has been studied for many years (Nishino and Koizumi, 1977; Zatz and Brownstein, 1979; Earnest and Turek, 1985), but there are still uncertainties about the origins of the cholinergic input to the SCN and the mechanisms of its action. We demonstrated that there are cholinergic projections to the hamster SCN originating from

several structures, including from two cholinergic nuclei in the pontine tegmentum and from portions of the forebrain cholinergic system (Bina et al., 1993). We found that many cells in the forebrain that project to the SCN and are immunoreactive for choline acetyltransferase (the synthetic enzyme for ACh) also contain low-affinity p75 nerve growth factor receptors (p75-NGFR). Projections from cells in the ganglion cell layer of the retina are another source of p75-NGFR in the SCN (Bina et al., 1997).

Carbachol, a non-specific cholinergic agonist, has been shown to phase shift circadian rhythms when injected into the cerebral ventricles or the SCN, but the mechanisms of its actions remain unclear. Part of the reason for this controversy has been that the endpoints and species examined have differed among studies. Thus, it appears that carbachol acts via receptors that resemble neuronal nicotinic receptors to shift pineal rhythms in rats (Zatz and Brownstein, 1981; Zatz and Brownstein, 1979), but evidence for a similar mechanism mediating the effects of carbachol on behavioral rhythms in hamsters (Keefe et al., 1987) is subject to alternative interpretations (Rusak and Bina, 1990; Bina and Rusak, 1996a). We re-examined this question and found that the muscarinic receptor antagonist atropine could block phase shifts of hamster activity rhythms induced by carbachol injections into the SCN, while the nicotinic antagonist mecamylamine was ineffective (Bina and Rusak, 1996a). Using a novel receptor binding method to reveal available muscarinic receptors, we also showed that the SCN and surrounding hypothalamus have muscarinic receptors, although at a modest density compared to regions like the striatum and cortex (Bina et al., 1998). The conclusion that muscarinic receptors principally mediate carbachol effects on circadian rhythms in hamsters has been reinforced by evidence for a similar mechanism mediating carbachol's phase-shifting effect on the rat SCN in a slice preparation (Liu and Gillette, 1996).

We also found that injections of nerve growth factor into the SCN caused phase shifts of circadian rhythms in hamsters, which resembled those caused by carbachol (Bina and Rusak, 1996b). We hypothesize that these shifts are mediated by alterations in cholinergic transmission in the SCN, or by induction of immediate-early gene expression which mimics the patterns of gene expression induced by photic stimuli in SCN cells.

Results from another laboratory have suggested that cholinergic inputs to the SCN might operate by presynaptically modulating release of other neurotransmitters. Carbachol-induced phase shifts were attenuated by pretreatment with a glutamatergic antagonist, suggesting that carbachol might act in part by regulating presynaptically the release of glutamate, perhaps from retinal ganglion cells (Colwell et al., 1993). As mentioned earlier, there are other glutamatergic inputs to the SCN which might be relevant (Moga and Moore, 1996), but the general principle is at least consistent with evidence that cholinergic projections can act presynaptically in other parts of the visual system and elsewhere (Aoki and Kabak, 1992; Henley et al., 1986; Wonnacott, 1997). It remains possible that there are both pre- and postsynaptic cholinergic receptors in the SCN, but the details of their localization and function are unknown.

In recent neurophysiological studies in rat hypothalamic slice preparations, we began to investigate the role of agonists and antagonists specific to cholinergic receptor subtypes in altering firing rates of SCN cells, when applied by microiontophoresis. Both carbachol and ACh had mixed effects on SCN cell firing rates, but most cells were suppressed by their application. Pirenzepine, a specific M1 muscarinic antagonist, was highly effective in blocking the activating effects of carbachol on 5/6 cells tested (83-100% blockade), but it was much less effective in blocking the more common suppressive effects of carbachol. Thus, it weakly potentiated the effects of carbachol on 2/15 cells and blocked only 45% of the effect of carbachol on the other 13 cells. Nicotine had very potent and consistent effects, activating almost all cells and suppressing only a very few others. We have not yet tested the effects of specific nicotinic antagonists (Ying and Rusak, unpublished).

These observations indicate that multiple cholinergic receptors mediate the effects of ACh on the SCN, but they do not address where these actions occur. It remains unclear whether ACh acts both pre- and postsynaptically, and what ultimate effects its release has on neurotransmission in the SCN. We have, therefore, also begun to investigate whether nicotine-induced activations are prevented by co-application of an NMDA antagonist (AP-5), as would be predicted if these effects of nicotine are mediated transsynaptically by increasing the release of glutamate from terminals in

the SCN. The results so far are consistent with this hypothesis and with the evidence that phase-shifting effects of intraventricular carbachol are prevented by blocking NMDA receptor activity (Colwell et al., 1993). However, a small number of firing-rate suppressions to nicotine application were not antagonized by AP-5 administration, suggesting that these effects may be directly on SCN cells or may be mediated by release of a neurotransmitter other than glutamate.

D. Serotonin (5-HT)

The receptors involved in mediating the effects of 5-HT in the SCN itself remain uncertain. Earlier work from our laboratory and others suggested a major role for 5-HT_{1A} receptors (Prosser et al., 1993; Ying and Rusak, 1994). Cloning of the 5-HT_7 receptor, however, and demonstration that many ligands with high affinity for 5-HT_{1A} receptors also have high affinity for 5-HT_7 receptors (including 8-OH-DPAT) reopened this issue (Lovenberg et al., 1993; Ruat et al., 1993). We have recently published a report on neurophysiological studies in vivo using antagonists with different affinities for these two receptor subtypes. Our results favor the conclusion that 5-HT_7 receptors mediate most of the inhibition of photic responses resulting from application of 5-$HT_{1A/7}$ agonists to photically responsive SCN neurons (Ying and Rusak, 1996; Quintero and McMahon, 1998).

These findings do not, of course, rule out an additional role for 5-HT_{1A} receptors in the SCN. In fact, 5-HT also acts through other receptors to affect SCN cells. 5-HT_{1B} receptors appear to be located presynaptically on retinal afferents to the SCN where their activation inhibits photic responses, presumably by reducing release of glutamate (Pickard and Rea, 1997).

The physiology of 5-HT's action on the circadian system is obviously complicated. The combination of pre- and postsynaptic receptors, targets in the IGL and SCN (which also reciprocally innervate each other), inhibitory autoreceptors in the raphe nuclei, and two apparently distinct but interacting projection routes from the raphe to the major circadian system components, create a complex puzzle. The mechanisms by which 5-HT alters photic effects on rhythms (Weber et al., 1998; Bradbury et al., 1997) may be very different from those which mediate phase-shifting effects of 5-HT agonists at a different phase (Starkey, 1996). Neurophysiological analyses of these several projection routes and of the types of receptors involved in each are needed in order to better understand these mechanisms.

We have begun this analysis with a series of studies of the effects of electrical and chemical stimulation of raphe neurons while recording responses in the IGL (and adjacent ventral LGN [vLGN]) and SCN of rats. Our first study involved electrical stimulation of the dorsal raphe in rats, intending to activate the projection to the IGL and assess the effects on spontaneous activity and on photic responses of IGL/vLGN neurons. In these studies, we found that among photically responsive IGL cells ~85% were activated by light, and the remainder suppressed. Periods of electrical stimulation in the raphe using a bipolar electrode suppressed firing rates of most photically responsive IGL cells tested (77%). Among photically responsive cells, the amplitudes of responses (primarily activations) to light were decreased during raphe stimulation in 8/10 cells. By contrast, among 21 dLGN cells tested under the same conditions, 11 showed increased and 10 decreased responses to light during raphe stimulation (Jiao and Rusak, unpublished observations). The observation of raphe activation suppressing photic responses in most IGL cells is consistent with the hypothesis that the stimulation releases serotonin in the IGL, inhibiting IGL cells and reducing their responsiveness to photic input. Since few cells have so far been tested and fewer still of the less common light-suppressed IGL cells, this interpretation is tentative.

E. Circadia

To facilitate collection and analysis of activity rhythm data from rodents, we have worked with Dr. Ralph Mistlberger to develop a Matlab-based, Macintosh-compatible data analysis system, Circadia. This project has been in development for several years, and a prototype beta-release version is now available and being tested in our laboratory.

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